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Quantification of cell lysis during CHO bioprocesses: Impact on cell count, growth kinetics and productivity

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ABSTRACT

High cell densities and high viability are critical quality attributes for mammalian bioprocesses. Determination of living and dead cell numbers is nowadays routinely performed by automated image-based cell analyzers or flow cytometry. However, complete lysis of cells is usually neglected by these devices. We present a novel method for robust quantification of lysed cell populations over the course of a CHO bioprocess. The release of lactate dehydrogenase (LDH) and double stranded genomic DNA in culture supernatants were used as markers for cell lysis. We considered the degradation of both markers over cultivation time, which significantly increased the amount of released LDH and DNA. For correct and robust estimation of lysed cell fractions, degradation of both markers over cultivation time was considered, where redundancy of markers allowed data reconciliation. Calculating the number of cells which were subject to complete cell lysis, we could show that this fraction makes up as much as 30% of the total produced biomass and is not described by measurements of image-based analyzers. Finally, we demonstrate that disregarding cell lysis heavily affects the calculation of biomass yields and growth rates and that increasing levels of cell lysis are related to decreased productivity.

1. Introduction

Chinese hamster ovary (CHO) cells represent the most widely applied mammalian host system for the biopharmaceutical production of therapeutic proteins (Jayapal et al., 2007; Li et al., 2010). In this context, cell death is a topic of major importance during any CHO bioprocess. Unfavorable cultivation conditions, accumulation of (toxic) byproducts or nutrient limitations can cause apoptosis and cell death (Krampe and Al-Rubeai, 2010). Low viability and low cell densities are the result which in turn affects product titers and also product quality, critical parameters for biopharmaceutical process performance (Birch and Racher, 2006). Moreover, increasing numbers of dead cells will also increase impurities, which again affects product quality and can complicate downstream processing (Birch and Racher, 2006; Liu et al., 2010).

Detection of dead cells can be performed in multiple ways using different methods and devices. However, the requirements on the applied methods are always the same. The methods must be (i) robust to different media/environmental influences, (ii) easy to perform in routine application, (iii) fast and also applicable for high-throughput measurements, which also requires a certain potential for automation.

Devices based on image-based cell analyses are among the most commonly applied for estimation of cell numbers. Image analysis can be performed completely automated nowadays, yielding total cell counts with a low error in only minutes. Moreover, discrimination between dead and living cells is possible using special dyes like trypan blue, propidium iodide or acridine orange. The application of such devices has emerged as routine analytics in recent years, especially for mammalian bioprocess development (Sonnleitner, 2013). However, complete lysis of previously died cells cannot be recorded by these devices, which can result in severe underestimation of dead cell numbers, especially during longer bioprocesses (Jaenisch et al., 2002).

Determination of both dead and lysed cell fractions can be achieved by monitoring the accumulation of death markers in culture supernatants. Lactate dehydrogenase (LDH) is surely one of

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the most prominent markers for estimation of cell death in mammalian cell culture (Legrand et al., 1992; Loo and Rillema, 1998). LDH is a sensitive and selective marker and is routinely applied during mammalian cultivations studies and for bioprocess development (Allen et al., 1994; Flickinger, 2013; Goergen et al., 1993). Methods based on detection of other enzymes of the central carbon metabolism or estimation of genomic DNA release often yielded unsatisfactory results and never established as standard analytics (Bernheim et al., 1977; Jaenisch et al., 2002; Kepp et al., 2011). A big disadvantage of these methods is the degradation of the death markers over time. While this issue is discussed in literature with respect to the validity of the estimated dead cell numbers (Minor, 2006), to our best knowledge, no publication gives clear instructions on how to deal with marker degradation for reliable estimation of cell death and cell lysis.

In the present publication, we aim to answer the question, how cell lysis can be quantified in a reliable way and how a typical CHO bioprocess is actually affected by cell lysis with regard to biomass and product formation. We will (i) present a high-throughput methodology for robust and sensitive estimation of dead and lysed cells from measurements of extracellular LDH and genomic DNA which considers marker degradation, (ii) use this methodology to quantify the amount of cell lysis during different CHO bioprocesses and (iii) demonstrate the impact of cell lysis on the calculation of physiological descriptors and the evaluation of process performance with regard to productivity.

2. Material and methods

2.1. Cell line, cultivation conditions and cell counting

An industrial CHO cell line producing a therapeutic monoclonal antibody (mAb) was cultivated in chemically defined medium. Batch cultivations were performed in 2 L scale bioreactors at 37 °C. Oxygen concentration was kept between 25% and 40% while pH was kept constant at 6.8, 7.0 or 7.2 by addition of 0.5 N NaOH and 0.5 N HCl. Cell counting was performed using the automated picture analyzer Cedex HiRes Analyzer (Roche Lifesciences, Mannheim, Germany). Briefly, cells were counted automatically by the device resulting in an average total cell count (TCC) from 10 independent pictures. Trypan blue staining of dead cells was used to distinguish between live cell count (VCC) and dead cell count (DCC).

2.2. Analysis of mAb, metabolites, LDH and DNA in culture supernatants

Cultivation samples were taken every 12 h and cells separated from culture supernatants by centrifugation for 10 min at 3000 × *g* and 4 °C. Glucose, glutamine, lactate, mAb and LDH in culture supernatants were determined by photometric assays using the automated enzymatic analyzer Cedex Bio HT (Roche Diagnostics GmbH, Germany). Analysis of double strand DNA in culture supernatants was performed using the Quant-iT™ PicoGreen® dsDNA Assay Kit (Thermo Fisher Scientific, Germany). Briefly, culture supernatants were diluted 50- to 100-fold, a standard calibration curve prepared using λ-DNA in the range of 4000–50 ng/mL and 5 μL of standard or sample mixed with 250 μL of freshly prepared assay reagent in a 96-well plate. Fluorescence intensity was monitored using a 96-well plate reader (Infinite® 200 PRO, Tecan Group Ltd, Switzerland) and DNA concentration in samples determined against the calibration standards. Triplicate measurements for DNA and LDH were performed to determine average values and standard deviations.

2.3. Evaluation of robustness and sensitivity of methods

For evaluation of the LDH assay, culture supernatants from the end of batch cultivations containing >2000 U/L of LDH were sequentially diluted to prepare a row of standards. Each standard was measured ten times and the mean values and standard errors determined. For DNA measurements, λ-DNA was diluted with cell culture media, to have a background comparable to the culture supernatant. These standards in the range of 4000–0.1 ng/mL were measured ten times each and mean values and standard errors determined. Statistical analysis for determination of signal to noise ratio (SNR), the limit of detection (LOD) and the limit of quantification (LOQ) was performed as described before (Wechselberger et al., 2013).

2.4. Degradation kinetics of LDH and DNA

Degradation rates were determined over the whole time course of the cultivation. At different time points during the cultivation, 2 mL cell-free culture supernatants were incubated in reaction tubes at 37 °C between 48 h and 72 h and sampled regularly to determine decrease of LDH activity and DNA concentrations over time as described in Section 2.2. Each degradation rate was determined in triplicates. The respective measurements of LDH and DNA were corrected for the respective degradation rates as shown in Eq. (1):

$$r_{corr} = -r_d + \frac{dc}{dt} \quad (1)$$

r_{corr}, corrected volumetric rate of LDH or DNA release [mU/(ml h)] or [ng/(mL h)]; r_d, volumetric rate of marker degradation [mU/(mL h)] or [ng/(mL h)]; dc/dt: change of marker concentration over time [mU/(mL h)] or [ng/(mL h)].

2.5. Cellular content of LDH and DNA

10 mL of cell suspension were harvested from bioreactors, and stored on ice. Cells were separated from culture supernatant as described in Section 2.2 and washed twice with 4 °C cold phosphate buffered saline (PBS). To avoid marker degradation by intracellular DNases or proteases, cells were resuspended in 10 mL PBS containing either 50 mM EDTA or protease inhibitors (cOmplete, Mini, EDTA-free, Roche Life Science, Germany). For cell breakup, cell suspensions were frozen at −20 °C for at least 1 h or overnight, thawed on ice and the remaining cells solubilized by mechanical disruption. Complete disruption of cells was controlled using the Cedex HiRes Analyzer. To completely inactivate DNases, cells suspensions in 50 mM EDTA supplemented PBS were additionally incubated for 10 min at 65 °C in a waterbath after breakup. Determination of DNA and LDH concentrations was performed as described in Section 2.2.

2.6. Estimation of lysed cell populations

LDH and DNA concentrations were corrected for respective degradation over time and the amount of corresponding cells calculated based on the average cellular content of LDH or DNA as shown in Eqs. (2) and (3):

$$r_{X,L} = \frac{(-r_d + (dc/dt))}{Y_{c/X}} \quad (2)$$

$$X_{lysis,n} = X_{lysis,n-1} + \frac{(r_{X,L,n} + r_{X,L,n-1}) * (t_n - t_{n-1})}{2} \quad (3)$$

$r_{X,L,n}$, volumetric rate of biomass lysis at time point n [cells/(mL h)]; t_n, time at time point n [h]; r_d, volumetric rate of marker degradation [mU/(mL h)] or [ng/(mL h)]; dc/dt, change of marker concentration over time [mU/(mL h)] or [ng/(mL h)]; $Y_{c/X}$ = cellular